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(54) Title: METHOD AND COMPOSITION FOR INHIBING OR SLOWING BLOOD COAGULATION

(57) Abstract: A method and composition for inhibiting or slowing blood coagulation includes lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.

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METHOD AND COMPOSITION FOR INHIBITING OR SLOWING BLOOD  
COAGULATION

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DEVELOPMENT

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**[0001]** The work leading to the present invention was supported by one or more grants from the U.S. Government, including National Heart, Lung, and Blood Institute Grant R01 HL57867. The U.S. Government therefore has certain rights in the invention.

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## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0002]** The present application claims priority on prior U.S. Provisional Application S.N. 60/386,562, filed June 7, 2002, and which is incorporated herein in its entirety by reference.

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## BACKGROUND OF THE INVENTION

[0003] The present invention is generally directed to inhibiting or slowing blood coagulation, and more particularly to using lactadherin or a  
5 fragment thereof as an agent for inhibiting or slowing blood coagulation.

[0004] There are several anticoagulant drugs which are in widespread clinical use and many others in development, including clinical trials. However, none of these agents have the mechanism of blocking  
10 access of blood proteins to procoagulant membrane surfaces. Inhibition of coagulation at this stage is an earlier step than most anti-coagulants target and, it is specifically an anticoagulant step as opposed to a step which would inhibit platelet aggregation or adhesion.

15 [0005] Investigators have sought anticoagulants that would work via this mechanism for decades. Phospholipases from snake venoms can function as anticoagulants by competing for membrane binding sites and the function of several phospholipases have been analyzed in detail. These proteins are unsuitable for use in humans because the corresponding  
20 enzymatic activity (phospholipases cleave phospholipids releasing lysophospholipids and free fatty acids) cause inflammation and tissue injury. A single class of agents, annexins, have been shown to inhibit blood

coagulation by blocking the membrane surface. These proteins have been evaluated as anticoagulants in animals. The efficacy is modest, at least partly because annexin is more fastidious in its requirements for membrane lipids than most blood clotting proteins. Thus, much of the procoagulant membrane of cells remains unblocked, even with a vast excess of annexin V. We compared the anticoagulant efficacy of annexin V directly to that of lactadherin.

[0006] Lactadherin is a MW 47,000 glycoprotein of milk fat globules. It has also been known as PAS-6/7, indicating the two glycosylation variants (Reference 1), bovine-associated mucoprotein, BA-46, P47, and MFG-E8 (Reference 2). Lactadherin has a domain structure of EGF1-EGF2-C1-C2 in which EGF indicates epidermal growth factor homology domains, and the C domains share homology with the discoidin family including the lipid-binding "C" domains of blood coagulation factor VIII and factor V (Figure 1) (Reference 2). The second EGF domain displays an Arg-Gly-Asp motif (Reference 3) which binds to the  $\alpha_v\beta_5$  and  $\alpha_v\beta_3$  integrins (References 1 and 4-6). The second C domain binds to phospholipids (Reference 6).

[0007] In milk fat globules, lactadherin lines the surface of the phospholipid bilayer which surrounds the central triglyceride droplet, apparently stabilizing the bilayer (Reference 7). Lactadherin decreases the

symptoms of rotavirus infection in infants, possibly by binding to rotavirus particles via carbohydrate moieties (Reference 8). In tissue sections, lactadherin is found localized on the apical portion of secretory epithelium in the breast (Reference 7). Abundant expression by breast carcinoma tissue  
5 makes lactadherin a potential target for antigen-guided radiation therapy (Reference 9). Lactadherin is also found on the apical surface of epithelia in the biliary tree, the pancreas, and sweat glands (Reference 7) and is synthesized by aortic medial smooth muscle cells (Reference 10). Function in these tissues remains unknown. Lactadherin has been identified as a zona  
10 pellucida-binding protein on the acrosomal cap of sperm (Reference 11).

[0008] Blood coagulation factor VIII and factor V bind to phospholipid membranes via "C" domains which share homology with lactadherin (References 12-14). Remarkable features of membrane binding  
15 for these proteins include high affinity ( $K_D$  approx. 2 nM) (Reference 15) and sufficient specificity so that no plasma proteins compete for membrane binding sites (Reference 16). Factor VIII binds via stereo-selective interaction with the phospho-L-serine motif of phosphatidylserine (PS) (Reference 17). Factor V also exhibits stereoselective interaction with PS (Reference 18).  
20 Binding of factor VIII is enhanced by the presence of phosphatidylethanolamine (PE) in the membrane (Reference 19), by unsaturated phospholipid acyl chains (Reference 20), and by membrane curvature (Reference 19). The crystal structures of the C2 domains of factors

VIII and V suggest that membrane binding is mediated by two pairs of hydrophobic residues displayed at the tips of  $\beta$ -hairpin turns (References 21-22). Mutagenesis studies have confirmed the role of these residues in phospholipid binding (Reference 23). The homology of the lactadherin C domains with those of factors VIII and V suggests that similar phospholipid binding properties may exist. Indeed, lactadherin has been found to bind selectively to PS (Reference 24) and to utilize primarily the C2 domain in its lipid binding (Reference 6).

10           **[0009]**           Annexin V, like factor VIII and factor V, exhibits high affinity, PS-dependent membrane binding (Reference 25). However, the quadruplicate membrane-binding motifs of annexin V are not homologous with the discoidin-like domains of lactadherin, factor VIII, and factor V (Reference 26). Corresponding to the difference in structure, the membrane binding mechanism is different. In addition, annexin V requires  $\text{Ca}^{++}$  for membrane-binding and the binding is chiefly hydrophilic in nature (Reference 27). Annexin V does have the capacity to compete for a fraction of the phospholipid binding sites utilized by the factor VIIIa-factor IXa enzyme complex and the factor Xa-factor Va enzyme complex of the coagulation cascade so that it functions *in vitro* as a membrane-blocking anticoagulant (Reference 28). The well-defined membrane-binding and anti-coagulant properties of annexin V make studies with annexin V suitable controls for studies with lactadherin.

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## OBJECTS AND SUMMARY OF THE INVENTION

**[0010]** The principal object of the present invention is to provide an agent which inhibits or slows blood coagulation. The agent includes  
5 lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.

**[0011]** An object of the present invention is to provide an agent which inhibits or slows blood coagulation by competing for membrane binding  
10 sites of factor VII and/or factor V.

**[0012]** Another object of the present invention is to provide an agent which inhibits or slows blood coagulation by inhibiting the prothrombinase complex and the factor VII a-tissue factor complex.  
15

**[0013]** Yet another object of the present invention is to provide an agent which inhibits or slows clotting of whole blood.

**[0014]** Still yet another object of the present invention is to  
20 provide an agent which inhibits or slows coagulation reactions on cell membranes.

[0015] An additional object of the present invention is to provide an agent which is an efficient inhibitor of the prothrombinase and the factor Xase complex regardless of the degree of membrane curvature and/or the phosphatidylserine content.

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[0016] Yet an additional object of the present invention is to provide an agent which is more effective than annexin V in inhibiting or slowing blood coagulation or clotting.

10 [0017] Lactadherin, a glycoprotein of the milk fat globule membrane, contains tandem C- domains with homology to discoidin-type lectins and to membrane-binding domains of blood-clotting factors V and VIII. We investigated whether the structural homology confers the capacity to compete for the membrane-binding sites of factor VIII and factor V and to  
15 function as an anticoagulant. Our results indicate that lactadherin competes efficiently with factor VIII and factor V for binding sites on synthetic phosphatidylserine-containing membranes with half-maximal displacement at lactadherin concentrations of 1-4 nM. Binding competition correlated to functional inhibition of factor VIIIa-factor IXa (factor Xase) enzyme complex. In  
20 contrast to annexin V, lactadherin was an efficient inhibitor of the prothrombinase and the factor Xase complexes regardless of the degree of membrane curvature and the phosphatidylserine content. Lactadherin also



inhibited the factor VIIa-tissue factor complex efficiently whereas annexin V was less effective. Since the inhibitory concentration of lactadherin was proportional to the phospholipid concentration, and because lactadherin was not an efficient inhibitor in the absence of phospholipid, the major inhibitor effect of lactadherin relates to blocking phospholipid sites rather than forming inhibitory protein-protein complexes. Lactadherin was also found to be an effective inhibitor of a modified whole blood prothrombin time assay in which clotting was initiated by dilute tissue factor; 60 nM lactadherin prolonged the prothrombin time 150% vs. 20% for 60 nM annexin V. These results indicate that lactadherin functions as a potent phospholipid-blocking anticoagulant.

**[0018]** At least one of the above-noted objects is met, in part, by the present invention, which in one aspect includes blocking or reducing access to a procoagulant molecule by a coagulation molecule by subjecting a procoagulant molecule to lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.

**[0019]** Another aspect of the invention includes blocking or reducing binding of a ligand to cell membrane binding site by subjecting a cell membrane binding site to lactadherin, a fragment of lactadherin, a functional

equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.

5           **[0020]**       Another aspect of the invention includes inhibiting or slowing blood coagulation by subjecting a predetermined amount of blood to an effective amount of lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.

10           **[0021]**       Another aspect of the invention includes inhibiting or slowing blood clotting by administering to a subject in need thereof an effective amount of lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.

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**[0022]**       Another aspect of the invention includes preventing or reducing inflammation by administering to a subject in need thereof an effective amount of lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.

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**[0023]**       Another aspect of the invention includes inhibiting or slowing blood coagulation by administering to a subject in need thereof an

effective amount of lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.

5           **[0024]**       Another aspect of the invention is to provide an anticoagulant reagent which includes lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin. A pharmaceutical composition may be prepared by utilizing the anticoagulant reagent with a pharmaceutically acceptable carrier or diluent.

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**[0025]**       Another aspect of the invention includes removing a phospholipid from a biological fluid by providing a biological fluid including, or suspect of including, a phospholipid, subjecting the biological fluid to a suitable amount of a binding agent (lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin) allowing binding between the phospholipid and the binding agent, and removing the binding agent with the phospholipid bound thereto.

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## BRIEF DESCRIPTION OF THE DRAWINGS

[0026] The above and other objects, novel features and advantages of the present invention will become apparent from the following  
5 detailed description of the invention, as illustrated in the drawings, in which:

[0027] Figures 1A-C illustrate competition for factor VIII and factor V binding sites by lactadherin or annexin V;

10 [0028] Figures 2A-F illustrate the relationship of vesicle curvature and PS content to inhibition of the prothrombinase complex by lactadherin or annexin V;

[0029] Figures 3A-F illustrate the relationship of vesicle  
15 curvature and PS content to inhibition of the factor Xase complex by lactadherin or annexin V;

[0030] Figures 4A-B illustrate inhibition of the factor VIIa-tissue factor complex by lactadherin or annexin V;

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[0031] Figure 5 illustrates competition for factor IXa binding sites by lactadherin;

[0032] Figures 6A-C illustrate the relationship of inhibition by  
5 lactadherin to phospholipid concentration; and

[0033] Figure 7 illustrates inhibition of whole blood prothrombin time by lactadherin or annexin V.

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## DETAILED DESCRIPTION OF THE INVENTION

[0034] The present invention is based, at least in part, on the discovery that lactadherin, a glycoprotein of milk fat globules, functions as an anticoagulant by competing with factors VIII and V for membrane binding  
15 sites. Our experiments below show that lactadherin functions as a potent phospholipid blocking anticoagulant.

### Materials

[0035] Human factor X, human factor Xa, and human factor IXa  
20 were obtained from Enzyme Research Laboratories (Southbend, IN), human factor V, human factor Va, and corn trypsin inhibitor were obtained from

Heamatologic Technologies Inc. (Burlington, VT). Recombinant human factor VIII was a gift from D. Pittman of Genetics Institute, Cambridge, MA. Human factor VIIa, human prothrombin, and human  $\alpha$ -thrombin were obtained from Enzyme Research Laboratories (South Bend, IN). Both recombinant human  
5 tissue-factor and lipidated recombinant human tissue-factor were obtained from American Diagnostica Inc. (Greenwich, CT). Lactadherin was a gift from Drs. C.W. Heegaard and J.T. Rasmussen of the Department of Molecular and Structural Biology, University of Aarhus, Denmark. Annexin V was obtained from Sigma. Bovine brain PS, egg yolk PE, and phosphatidylcholine (PC)  
10 were obtained from Avanti Polar Lipids (Alabaster, AL). Chromogenic substrate S-2238 and S-2765 were obtained from DiaPharma Group Inc (Westchester, OH).

## Methods

### 15 Evaluation and Storage of Proteins

[0036] Pure bovine lactadherin was supplied at a concentration of 1 mg/ml in phosphate-buffered saline. SDS-PAGE with silver staining revealed only two bands corresponding to the previously described lactadherin doublet with approximate MW's of 47 kDa and 50 kDa.  
20 Lactadherin was stored at  $-80^{\circ}\text{C}$ , aliquotted after thawing and each aliquot subjected to less than 3 cycles of flash-freezing and rapid thawing. The purity and handling of other proteins were as previously described (Reference 29).

### Preparation of Phospholipid Vesicles

[0037] Phospholipid vesicles were prepared by evaporating chloroform from the desired phospholipids (PS%: PE%: PC% = 4:20:76 and 15:20:65), resuspending in methylene chloride and re-evaporating twice under argon. Phospholipids were then suspended by gently swirling tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) over the dried lipid suspension until all lipid was suspended. Vesicles prepared in this way were used as large multilamellar vesicles (LMV) (Reference 30). Some of the resuspended vesicles were then sonicated in a high intensity bath sonicator (Laboratory Supplies, Inc. Hicksville, NY) (Reference 31) and some were made extruding these phospholipid suspensions twenty times through two stacked polycarbonate membranes (Millipore, Bedford, MA) in a High Pressure Extrusion Device (Sciema Technical Services, Vancouver, BC, Canada) under argon as described previously (Reference 32). Phospholipid concentration was determined by phosphorus assay (Reference 33). Vesicles were used fresh, or 1 ml aliquots were quick-frozen in liquid nitrogen, stored at -80 °C, and thawed at 37 °C. Storage at 4 °C before incubation with microspheres or blood clotting factors did not exceed 24 hours.

### 20 Relipidation of TF

[0038] Recombinant human tissue factor was relipidated into phospholipid vesicles of the indicated composition using the octyl- $\beta$ -D-

glucopyranoside method (Reference 34). The nominal molar ratios of TF to phospholipid monomer was 1:7500.

#### Fluorescein-Glu-Gly-Arg Chloromethyl Ketone Labeling of Factor IXa

5           **[0039]**       Factor IXa was labeled with fluorescein-Glu-Gly-Arg chloromethyl ketone (Haematologic Technologies, Burlington, VT) essentially as described for the Dansyl-Glu-Gly-Arg chloromethyl ketone (Reference 35). Free fluorescein-peptide was removed by ultrafiltration (Centricon 30, Millipore Corp., Bedford, MA). Labeling efficiency, as judged by the ratio of  
10 absorbance at 280 nm to absorbance at 490 nm, divided by extinction coefficients for factor IXa and fluorescein, was 0.2 fluorescein-peptide:factor IXa.

#### Flow Cytometry Binding Assay

15           **[0040]**       Lipospheres were prepared as previously described (Reference 16). Glass microspheres of 1.6  $\mu\text{m}$  nominal diameter (Duke Scientific, Palo Alto, CA) were cleaned, size-restricted, incubated with sonicated vesicles of the indicated composition, washed three times in 0.15 M NaCl, 0.02 M Tris-HCl, 0.1% defatted bovine albumin, 10  $\mu\text{M}$  egg PC as  
20 sonicated vesicles. Lipospheres were stored at 4 °C and used within 8 hours of synthesis. Recombinant human factor VIII and purified human factor V were labeled with fluorescein maleimide as described (Reference 36).



Fluorescein-labeled factor VIII (4 nM) or fluorescein-labeled factor V(4 nM) were incubated with lactadherin or annexin V for 15 min at room temperature, the mixture was added to lipospheres for an additional 10 minutes, and membrane-bound fVIII or factor V was measured by flow cytometry. This  
5 procedure was performed on 25- $\mu$ L aliquots of 100- $\mu$ L samples with an approximate liposphere concentration of  $1 \times 10^6$ /mL using a Becton Dickinson FACSCalibur flow cytometer. Data acquisition was triggered by forward light scatter with all photomultipliers in the log mode. Noise was reduced during analysis by eliminating events with forward and side scatter values different  
10 from those characteristic of the lipospheres. Mean log fluorescence was converted to linear fluorescence for values depicted in the figures. Only experiments in which the fluorescence histogram indicated a log normal distribution, as judged by inspection, were analyzed quantitatively. Flow cytometry experiments were performed in 0.14 M NaCl, 0.02 M Trizma-HCl,  
15 1.5 or 5 mM  $\text{CaCl}_2$  as indicated in figure legends, and 0.1% bovine albumin, pH 7.5.

## Mathematical Model

[0041] Competition of lactadherin for phospholipid binding sites of factor VIII was compared to the following model:

$$fVIII B / fVIII B(\max) = [fVIII] / ((K_D * (1 + [Lactadherin] / K_i)) + [fVIII])$$

5 where fVIII B is membrane-bound factor VIII, fVIII B(max) is maximum bound factor VIII when the concentration of factor VIII is saturating,  $K_D$  is the dissociation constant of factor VIII with phospholipid binding sites,  $K_i$  is the dissociation constant of lactadherin with the binding sites recognized by factor VIII. The model assumes that phospholipid binding sites are limiting. For the  
10 curves depicted in Figures 1-3, all data was normalized to the calculated value of fVIII B/fVIII B(max) at the indicated concentrations of factor VIII without lactadherin. For factor Xase complex activity fVIII B/fVIII B(max) was assumed to be proportional to residual activity. Curve fitting was by eye, while varying  $K_i$ , as the concentrations of factor VIII and lactadherin were known and the  
15  $K_D$  value for factor VIII (Reference 30) had been determined experimentally (see Figures 1A-C, where displayed results are from a single experiment representative of at least two experiments for all conditions). The same model, substituting fV for fVIII, was used for comparison to competition binding experiments with factor V and inhibition of the prothrombinase  
20 complex.

### Factor Xase Assay

[0042] The activation of factor X by factor IXa in the presence of lactadherin or annexin V was measured with a two-step amidolytic substrate assay (Reference 29) with the following modifications. Factor IXa, 0.1 nM, was incubated with the specified concentrations of factor X and varied concentrations of lactadherin and annexin V in 150 mM NaCl, 50 mM Tris, pH 7.4, 1.5 mM  $\text{CaCl}_2$  (for 4% PS, sonicated vesicle) or 5 mM  $\text{CaCl}_2$  (for the other vesicles), 1 nM factor VIII, 0.1 nM thrombin for 5 min. at 25 °C; final reaction volume, 40  $\mu\text{l}$ . The reaction was then stopped by the addition of EDTA to 7 mM final concentration. The amount of factor Xa generated was determined immediately using the chromogenic substrate S-2765 (0.31 mg/mL) on a Molecular Devices ELISA plate reader in kinetic mode. The results displayed in the figures are means of duplicates from a representative experiment. For studies without phospholipid, 40 nM factor IXa was mixed with 40 nM factor VIII and 250 nM factor X in the reaction buffer. The reaction was started by addition of 2 nM thrombin and 1.5 mM  $\text{Ca}^{++}$ . The reaction was allowed to proceed for 30 min. prior to quenching with EDTA and reading, as above.

### Factor VIIa-Tissue Factor Assay

[0043] Relipidated tissue factor, at the indicated concentration, was mixed with factor X (100 nM) factor VIIa (100 pM) and varied concentrations of lactadherin or annexin V. The reaction was started by

addition of 1.5 mM  $\text{CaCl}_2$  and allowed to proceed for 5 min. at 25 °C. The reaction was stopped with EDTA and the quantity of factor Xa formed was determined as described above for the factor Xase assay.

## 5 Prothrombinase Assay

[0044] Cleavage of prothrombin to thrombin was measured in a two-step amidolytic substrate assay analogous to that for factor X activation as previously described (Reference 37). Factor Va, 1 nM; factor Xa, 6.2 pM; and lactadherin or annexin V at specified concentrations were incubated for 5 min. at 25 °C in a solution containing 150 mM NaCl, 50 mM Tris, 1.5 mM (for 4% PS, sonicated vesicles) or 5 mM (for the other PS content and/or vesicle size)  $\text{CaCl}_2$ , and 0.05% wt/vol ovalbumin, pH 7.8, prior to addition of 1  $\mu\text{M}$  prothrombin. After 5 min at 25 °C, the reaction was stopped by the addition of EDTA to a final concentration of 7 mM. Thrombin formation was assessed in a kinetic microplate reader immediately after addition of 0.1 mM chromogenic substrate S-2238. The results displayed in the figures are means of triplicates from a representative experiment.

## Activated Partial Thromboplastin Time and Prothrombin Time Assays

[0045] Pooled normal plasma, anticoagulated with 1:9 dilution of 3.8% citrate, was stored at -80 °C until usage. All reagents were pre-warmed to 37 °C and assays were performed in triplicate. For the activated partial

thromboplastin time (aPTT) assay 76  $\mu$ l of plasma was mixed with 76  $\mu$ l aPTT reagent (aPTT-SA, Helena Laboratories, Beaumont TX) and 76  $\mu$ l of lactadherin diluted into tris-buffered saline. After 10 min. 76  $\mu$ l of 25 mM  $\text{Ca}^{++}$  was added to start the clotting reaction. Time to fibrin strand formation was measured with a fibrometer. The aPTT reagent was either used at full strength or diluted 1:16 in 100  $\mu$ M ellagic acid, as indicated (to maintain a constant ellagic acid concentration as the phospholipid was diluted).

[0046] For the prothrombin time (PT) assay 100  $\mu$ l plasma was mixed with 100  $\mu$ l lactadherin in tris-buffered saline and 100  $\mu$ l PT reagent (Thromboplastin-C Plus, Baxter, Miami, FL). Because package instructions call for use of the PT reagent at a 2:1 ratio with plasma, the PT reagent was supplemented to 16 mM  $\text{Ca}^{++}$  to achieve the manufacturer's intended final  $\text{Ca}^{++}$  concentration. The PT reagent was also diluted 1:24 in 16 mM  $\text{Ca}^{++}$ , then utilized in the same manner as full strength PT reagent.

#### Whole Blood Prothrombin Time Assay

[0047] Blood was drawn from healthy, nonsmoking, non-aspirin-using volunteers using 19-gauge butterfly needles. The first 3-ml of blood was discarded. Subsequently, 20 ml of blood was gently drawn. The blood was rapidly discharged into a polypropylene tube containing sodium citrate, final concentration 10 mM. To suppress contact activation, corn trypsin inhibitor

was added to the tube (final concentration, 25  $\mu\text{g/ml}$ ). The blood was kept at room temperature, gently mixed by inversion approximately every 10 min. and was used within 3 hr of collection. For the clotting reaction 150  $\mu\text{l}$  aliquots of blood were pre-warmed to 37  $^{\circ}\text{C}$  for 10 min then diluted 1:1 with 10 mM  $\text{CaCl}_2$  and 50 pM relipidated tissue factor (0.38  $\mu\text{M}$  phospholipid). The time to fibrin strand formation was monitored with a fibrometer. Experiments were performed in triplicate.

## Results

10        **[0048]**        We hypothesized that the tandem C domains of lactadherin confer phospholipid binding properties that enable it to compete with factor VIII and/or factor V for membrane binding sites and function as an anticoagulant. To test this hypothesis, we performed a competition membrane binding experiment in which lactadherin competed with fluorescein-labeled  
15        factor VIII for membrane binding sites (Figure 1A).

**[0049]**        For the experiments illustrated in Figure 1A, fluorescein-labeled factor VIII, 4 nM, was mixed with the indicated concentration of competitor, lactadherin ( $\sigma$ ) or annexin V ( $\bullet$ ) in the presence of 1.5 mM  $\text{Ca}^{++}$ .  
20        Lipospheres were added and bound factor VIII was evaluated by flow cytometry after 10 min. Liposphere membrane composition was PS:PE:PC =

4:20:76. The inhibition curve was modeled assuming that the  $K_D$  of factor VIII with lipospheres was 4.8 nM, the  $K_i$  for lactadherin 0.5 nM (Reference 16).

[0050] The membranes had a composition of 4% PS, 20% PE, with the balance as PC. Bilayers were supported on 2  $\mu$ m diameter glass microspheres (lipospheres) and binding of factor VIII was evaluated by flow cytometry (Reference 16). Lactadherin effectively competed for all factor VIII binding sites with half-maximal displacement occurring at approximately 1.5 nM lactadherin. The competition predicted by a mathematical model approximated the data when lactadherin was assigned a  $K_i$  of 0.5 nM.

[0051] For comparison with lactadherin, we determined if annexin V would compete for factor VIII binding sites in the same assay (Figure 1A). Annexin V competed for approximately 20% of factor VIII binding sites at a concentration of 32 nM. These results indicate that lactadherin is a more potent competitor for the phospholipid binding sites of factor VIII on membranes with 4% PS.

[0052] To determine whether lactadherin is also able to recognize the phospholipid binding sites of factor V, we performed similar competition experiments with fluorescein-labeled factor V (Figure 1B).

[0053] For the experiments illustrated in Figure 1B, fluorescein-labeled factor V, 4 nM, was mixed with lactadherin ( $\sigma$ ) or annexin V ( $\bullet$ ) and bound factor V was evaluated under the conditions described above. The inhibition curve was modeled assuming that the  $K_D$  of factor V with lipospheres was 4.3 nM, the  $K_i$  for lactadherin, 1.0 nM (Reference 16).

[0054] Lactadherin competed efficiently for the binding sites recognized by factor V with half-maximal inhibition at an approximately 2-fold higher concentration. Annexin V competed for only 20% of the factor V binding sites at concentrations up to 32 nM. The corresponding  $K_i$  for a curve approximating the data was 1.0 nM.

[0055] We performed factor Xase assays in the presence of increasing concentrations of lactadherin to determine whether the competition for factor VIII binding sites would translate into inhibition of the factor Xase complex (Figure 1C).

[0056] For the experiments illustrated in Figure 1C, lactadherin ( $\sigma$ ) or annexin V ( $\bullet$ ) was mixed with factor IXa, 0.1 nM, and factor VIII, 1 nM, and factor X, 100 nM, prior to the addition of 1  $\mu$ M sonicated vesicles with 1.5 mM  $Ca^{++}$  and thrombin. The reaction was stopped after 5 min. and factor Xa was measured with chromogenic substrate S-2765 in a kinetic microplate



reader. The phospholipid composition of the sonicated vesicles utilized was the same as for the binding experiments depicted in Figure 1A. Lactadherin was an effective competitor for binding sites of factor VIII and factor V and potent inhibitor of the factor Xase complex with half-maximal inhibition at approximately 6 nM and > 98% inhibition at 32 nM. Annexin V was an ineffective inhibitor of the factor Xase complex with less than 10% inhibition at 64 nM annexin V. These results confirm that lactadherin is able to inhibit the factor Xase complex, most likely by competing with factor VIIIa and/or factor IXa and factor X for phospholipid binding sites. They do not, however, explain why annexin V, which also binds to phospholipid membranes with high affinity, is an ineffective inhibitor under these conditions.

[0057] We determined whether lactadherin is capable of inhibiting the prothrombinase complex, in a manner similar to the factor Xase complex (Figures 2 A-F, where displayed results are from a single experiment representative of either two or three experiments for all conditions). Prior reports indicate that membrane binding and anticoagulant efficacy of annexin V is related to the PS content of membranes and inversely related to the curvature of phospholipid membranes (Reference 38). Thus, we evaluated vesicles with maximal curvature (sonicated-nominal diameter of 20 nm (Reference 39)) intermediate curvature (extruded- nominal diameter  $73 \pm 25$  nm (Reference 32)) and minimal curvature (large multilamellar vesicles-

diameters >400 nm (Reference 30)) with both low PS content (4% PS- Figure 2A-C) and high PS content (15% PS- Figures 2D-F).

[0058] For the experiments illustrated in Figures 2A-F, sonicated  
5 ( $\sigma$  and  $\Delta$ ), extruded ( $\bullet$  and  $\mu$ ), and LMV ( $\blacksquare$  and  $\square$ ) contained 4% PS  
(Figures 2A-C) or 15% PS (Figures 2D-F). Effective concentrations of the  
vesicles for supporting the prothrombinase complex were identified in  
phospholipid titration experiments (Figures 2A and 2D). Subsaturing  
phospholipid concentrations were selected for inhibition experiments (open  
10 symbols in Figures 2A and 2D). The indicated phospholipid  
composition/concentration was added to factor Xa, factor Va, prothrombin,  
and either lactadherin or annexin V. After 5 min. the reaction was quenched  
with EDTA and thrombin measured with chromogenic substrate S-2238 in a  
kinetic microplate reader. Lactadherin was an effective inhibitor of the  
15 prothrombinase complex regardless of membrane curvature or PS content  
(Figures 2B and 2E). In contrast, inhibition by annexin V was inversely  
related to curvature and directly related to PS content (C, F). The  $\text{Ca}^{++}$   
concentration was 1.5 mM for sonicated vesicles of 4% PS ( $\sigma$  in A, B, C) and  
5 mM for all other conditions.

20

[0059] The effectiveness of the vesicles for supporting  
prothrombinase complex decreased as the curvature decreased with

sonicated vesicles of 4% PS producing 90 % of the maximum thrombin production at a concentration of 0.1  $\mu$ M phospholipid and much higher concentrations required for extruded vesicles and large multilamellar vesicles (LMV) (Figure 2A). We compared effects of lactadherin and annexin V on  
5 thrombin formation utilizing the phospholipid concentrations (indicated by open symbols in Figure 2A). We maintained the  $\text{Ca}^{++}$  concentration at 1.5 mM, equivalent to plasma, for sonicated vesicles with 4% PS to test the reproducibility of results in Figures 1A-C. However, we increased the  $\text{Ca}^{++}$  concentration to 5 mM for all other vesicles in order to optimize binding  
10 conditions for annexin V. Results showed that > 90% inhibition of thrombin production was effected by lactadherin in sonicated, extruded and LMV. Our results confirmed that sonicated vesicles provide a substantially more potent surface for the prothrombinase complex than vesicles of larger diameter, particularly when the PS content is 4%. In contrast, annexin V was ineffective  
15 for sonicated vesicles and reached only 25-40% inhibition on extruded vesicles and LMV at 64 nM.

[0060] Lactadherin was an efficient inhibitor of the prothrombinase complex on all vesicles containing 15% PS (Figure 2E). The  
20 half-maximal inhibitory concentrations of lactadherin varied with vesicle type. The limiting concentrations of phospholipids for experiments in Figure 2B facilitated comparison of actual inhibition to that predicted by a  $K_i$  of 1.0 nM, as depicted in Figure 1B. (The term " $K_i$ " is used to indicate only competition of

lactadherin for binding sites of factor factor V(a) or factor VIII(a) when phospholipid binding sites are limiting.) The inhibition curve approximates the data obtained with sonicated vesicles. The variation in the concentration of lactadherin required for different vesicle types (Figures 2B and 2E) could be

5 rationalized by assuming that lactadherin binds well to all types of phospholipid vesicles and that the higher phospholipid concentrations of extruded vesicles and LMV required more lactadherin to saturate the surface. In contrast to lactadherin, annexin V was only an effective inhibitor on LMV when the PS content was 15% (Figures 2C and 2F). Under these conditions

10 Annexin V inhibited approximately 90% of prothrombinase activity at 128 nM concentration, similar to results in a prior report (References 38 and 40). Annexin V was not as effective as lactadherin under any conditions evaluated. These results indicate that lactadherin efficiently inhibits the prothrombinase complex and that, in contrast to Annexin V, inhibition is not closely tied to PS

15 content or to vesicle curvature.

**[0061]** We determined whether inhibition of the factor Xase complex by annexin V and lactadherin may also be related to PS content of membranes and membrane curvature (Figures 3A-F, where data are from a

20 single set of experiments representative of two or three experiments for all conditions).

[0062] For the experiments illustrated in Figures 3A-F, sonicated ( $\sigma$  and  $\Delta$ ), extruded ( $\bullet$  and  $\mu$ ), and LMV ( $\blacksquare$  and  $\square$ ) contained 4% PS (Figures 3A-C) or 15% PS (Figures 3D-F). Effective concentrations of the vesicles for supporting the factor Xase complex were identified in phospholipid titration experiments (Figures 3A and 3D). Subsaturating phospholipid concentrations were selected for inhibition experiments (open symbols in Figures 3A and 3D). Lactadherin was an effective inhibitor of Xase activity regardless of membrane curvature or PS content (Figures 3B and 3E). In contrast, inhibition by annexin V was inversely related to curvature and directly related to PS content (Figures 3C and 3F). Experimental conditions were as those for experiments illustrated in Figures 1A-C. The  $\text{Ca}^{++}$  concentration was 1.5 mM for sonicated vesicles of 4% PS ( $\sigma$  in Figures 3A-C) and 5 mM for all other conditions.

[0063] Our results indicate that, like the prothrombinase complex, the factor Xase complex is supported at lower concentrations of sonicated vesicles than extruded vesicles or LMV (Figures 3A and 3D). Also, the inhibition of the Xase activity on sonicated vesicles was approximated by a model in which lactadherin is assigned a  $K_i$  of 0.5 nM (Figure 3B), similar to the value correlating to direct competition for factor VIII binding sites (Figure 1A). Lactadherin inhibited factor Xase complex more than 95% on all vesicle types, similar to inhibition of the prothrombinase complex (Figures 3B and 3E). However, the difference between the lactadherin concentrations required

for inhibition of the factor Xase complex on sonicated vs. extruded vesicles and LMV was not as large as for the prothrombinase complex (Figures 2B and 2E). Annexin V was a more effective inhibitor of the factor Xase complex than the prothrombinase complex with inhibition reaching 80% for LMV of 4% PS (Figure 3C) and 95% for LMV of 15% PS (Figure 3F). However, Annexin V remained a poor inhibitor of the factor Xase complex on sonicated vesicles, with < 20% inhibition for 4% PS and < 50% for 15% PS. Together these results indicate that lactadherin is a potent, near-complete inhibitor of the prothrombinase and factor Xase complexes on synthetic membranes regardless of membrane curvature and over a wide range of PS content.

[0064] Figures 4A-B illustrate inhibition of the factor VIIa-tissue factor complex by lactadherin or annexin V. Recombinant tissue factor was reconstituted into vesicles of composition PS:PE:PC, 4:20:76 by detergent dialysis with tissue factor:PL ratio of 1:7500. The indicated concentrations of phospholipid, with included tissue factor, were incubated with the indicated concentrations of lactadherin or annexin V, 100 pM factor VIIa, 100 nM factor X, and 1.5 mM  $\text{Ca}^{++}$  for 5 min prior to addition of EDTA and the chromogenic substrate S-2765. Lactadherin inhibited function of the factor VIIa-tissue factor complex efficiently (Figure 4A) while annexin V was less effective (Figure 4B). Displayed data with tissue factor concentration of 1 nM ( $\diamond$ ) are representative of three experiments. Data from experiments with a tissue

factor concentration of 0.2 nM ( $\mu$ ) and 0.04 nM ( $\sigma$ ) are representative of two experiments.

[0065] We determined whether Lactadherin might also have the capacity to inhibit the factor VIIa-tissue factor complex (Figure 4A). Recombinant tissue factor was reconstituted into vesicles containing 4% PS, 20% PE by dialyzing octylthioglucoside away from the tissue factor-phospholipid mixture. Vesicles prepared in this way have curvature comparable to extruded vesicles (Reference 41). Lactadherin inhibited the factor VIIa-tissue factor complex more than 90%. The quantity of lactadherin required for 50% inhibition varied with the tissue factor concentration, and corresponding phospholipid concentration. Annexin V was a weaker inhibitor of the factor VIIa-tissue factor complex (Figure 4B) with less than 50% inhibition at an annexin V concentration of 64 nM. These results indicate that lactadherin has the capacity to compete for membrane binding sites of blood coagulation proteins other than factor V and factor VIII.

[0066] To directly probe the possibility that lactadherin can compete with vitamin K-dependent blood coagulation proteins for membrane binding, we determined whether lactadherin is able to compete with fluorescein-labeled factor IXa (Figure 5).

[0067] For the experiments illustrated in Figure 5, the active site histidine of factor IXa was derivatized with fluorescein-Glu-Gly-Arg chloromethyl ketone as described under methods. Four nM fluorescein-labeled factor IXa was mixed with various concentrations of lactadherin or  
5 unlabeled factor IXa in the presence of 5 mM  $\text{Ca}^{++}$  prior to the addition of lipospheres and evaluation of bound fluorescein-labeled factor IXa as described for Figures 1A-C. Lactadherin and unlabeled factor IXa both competed with labeled factor IXa for membrane binding sites. Results are displayed with the abscissa on a log scale to enable direct comparison.  
10 Displayed data are representative of two experiments.

[0068] Fluorescein-labeled factor IXa bound to lipospheres with a dissociation constant of approx. 0.5  $\mu\text{M}$  (data not shown), consistent with prior reports of the membrane-binding affinity of factor IX/factor IXa.  
15 Unlabeled factor IXa competed with fluorescein-labeled factor IXa indicating that membrane binding was not enhanced by derivatization by fluorescein-Glu-Gly-Arg chloromethyl ketone at the active site. Lactadherin competed with fluorescein-labeled factor IXa for membrane binding sites to the same extent as unlabeled factor IXa. However, half-maximal inhibition occurred at  
20 approximately 4 nM lactadherin vs. 300 nM factor IXa.



[0069] In order to confirm that the mechanism through which lactadherin inhibits membrane-bound blood coagulation complexes is via competition for membrane binding sites, we performed experiments with varying phospholipid concentrations (Figures 6A-C).

5

[0070] For the experiments illustrated in Figures 6A-C, factor Xase complex (Figure 6A) and prothrombinase complex (Figure 6B) function on sonicated vesicles was evaluated at 0.1 (●), 0.5 (□), and 2.5 (σ) μM phospholipid concentrations in the presence of 1.5 mM CaCl<sub>2</sub>. Function of the factor Xase complex in the absence of phospholipid was also evaluated (Figure 6C). Inhibition by lactadherin at various concentrations was measured using two step amidolytic assays for the production of factor Xa (Figures 6A and 6C) or thrombin (Figure 6B) as described above. The inhibitory concentration of lactadherin was directly related to phospholipid concentration. In the absence of phospholipid (Figure 6C) lactadherin was an inefficient inhibitor of the factor Xase complex. Data obtained at various phospholipid concentrations were normalized for comparison of relative inhibition by lactadherin. Phospholipid composition was PS:PE:PC 4:20:76. Displayed data are from a single set of experiments (Figures 6A and 6B) or a single experiment representative of three experiments (Figure 6C).

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[0071] When the phospholipid vesicle concentration was limiting, lactadherin inhibited 50% activity of the factor Xase complex (Figure 6A) and the prothrombinase complex (Figure 6B) at a concentration of approx. 2 nM. The concentration required increased for each increment in the phospholipid concentration indicating that the required lactadherin is related to the phospholipid concentration rather than the concentrations of blood coagulation proteins. Furthermore, when the phospholipid concentration was 2.5  $\mu$ M the factor Xase complex and prothrombinase complex maintained more than 90% activity at 8 nM lactadherin, a concentration that inhibits activity more than 90% when the phospholipid concentration is limiting.

[0072] As noted above, we also evaluated the effect of lactadherin on the factor VIIIa-factor IXa complex in the absence of phospholipid (Fig 6C). The concentrations of factor VIIIa, factor IXa, and factor X were chosen to be at or below their apparent  $K_D$ 's or  $K_M$ , respectively, thus optimizing the sensitivity of the reaction to inhibitory action of lactadherin. Lactadherin caused less than 50% inhibition at concentrations up to 512 nM. Thus, lactadherin is at least 1,000-times better inhibitor in the presence of phospholipid membranes.

20

[0073] These results indicate that the only mechanism through which lactadherin inhibits the factor Xase and prothrombinase complexes at

the concentrations employed is by competitive occupation of phospholipid binding sites and that protein-protein complexes between lactadherin and factor VIIIa, factor Va, factor IXa, or factor X do not occur, or do not significantly inhibit function of these enzyme complexes.

5

[0074] To further investigate the dependence of lactadherin's inhibitory properties on phospholipid concentration, we evaluated inhibition of plasma clotting in activated partial thromboplastin time (aPTT) and prothrombin time (PT) assays. Commercial aPTT and PT reagents contain  
10 high concentrations of phospholipids of unknown or unspecified composition. Lactadherin inhibited the aPTT assay by approx. 10% at a concentration of 1,000 nM and had no effect at 100 nM or lower concentrations. However, when the aPTT reagent was diluted to 6% of the original concentration the aPTT was prolonged 5%, 20%, and 1000% at concentrations of 10, 100, and  
15 1,000 nM, respectively. Similarly, 1,000 nM lactadherin inhibited the prothrombin time by <20% when the PT reagent was present at 50% of manufacturer's suggested usage. However, when the PT reagent was diluted to 2% of the stock concentration the PT was prolonged 5%, 30%, and 500% by lactadherin concentrations of 10, 100, and 1,000 nM, respectively. These  
20 results are consistent with the model in which the major mechanism through which lactadherin inhibits blood coagulation enzyme complexes is through competition for phospholipid binding sites. Further studies, with defined phospholipid membranes are recommended to determine whether the

apparent  $K_i$ 's for inhibition of the isolated prothrombinase and Xase complexes correlate to inhibitory concentrations for plasma.

[0075] The results above, showing inhibition of the factor Xase,  
5 the prothrombinase and the factor VIIa-tissue factor complexes support the hypothesis that lactadherin would inhibit the rate at which whole blood clots. To test this prediction, we utilized a modified whole blood prothrombin time (Figure 7).

10 [0076] For the experiments illustrated in Figure 7, fresh whole blood was anticoagulated with 10 mM citrate and 25  $\mu$ g/ml corn trypsin inhibitor (to minimize activation of the intrinsic pathway prior to the prothrombin time assay) in a polypropylene tube. After addition of indicated quantities of lactadherin ( $\sigma$ ) or annexin V ( $\bullet$ ), the blood was diluted 1:1 with  
15 50 pM tissue factor and 10 mM  $\text{Ca}^{++}$ . Time to fibrin strand formation was measured with a fibrometer. Blood coagulation was initiated by simultaneous addition of calcium and 50 pM tissue factor prepared as in Figures 4A-B. Results are displayed as the ratio of prothrombin time for blood treated with annexin V or lactadherin to prothrombin time for control blood from the same  
20 donor, with the same elapsed time since phlebotomy. Displayed results are mean  $\pm$  SD for triplicate samples from a single experiment representative of three experiments.

[0077] In the absence of lactadherin or annexin V, the time to clot varied between 1200 and 2000 seconds for different donors. Lactadherin and annexin V led to prolongation of the clotting time and the prolongation was similar over a concentration range of 0-20 nM. However, at  
5 concentrations of 40 nM and above lactadherin led to progressively longer inhibition of blood clotting. The clotting time was prolonged approximately 3-fold at 100 nM lactadherin, but only about 1.5-fold by annexin V. These results indicate that Lactadherin competes for binding sites on cell membranes to inhibit blood coagulation in a manner similar to inhibition of  
10 isolated blood coagulation complexes on phospholipid vesicles.

[0078] Our results indicate that lactadherin binds to PS-containing membranes with sufficient affinity to compete with blood coagulation proteins. The phospholipid-binding competition makes lactadherin  
15 a potent inhibitor of the prothrombinase, the factor Xase, and the factor VIIa-tissue factor complexes of blood coagulation. Because the quantity of lactadherin necessary to inhibit these enzyme complexes is proportional to the phospholipid concentration employed, and because lactadherin does not efficiently inhibit the phospholipid-free factor VIIIa-factor IXa complex, the  
20 major mechanism of inhibition involves blocking of the phospholipid surface rather than formation of inhibitory protein-protein complexes. Inhibition of whole blood prothrombin time indicates that lactadherin binds to platelet

membranes to inhibit blood coagulation in a mechanism similar to inhibition of reconstituted enzyme complexes on phospholipid vesicles.

[0079] The enzyme complexes of blood coagulation assemble  
5 and function efficiently only on a membrane surface. PS-containing  
membranes serve to increase the apparent affinity of the respective cofactors,  
factor VIIIa and factor Va, for the enzymes, factor IXa and factor X, and of the  
cofactor-enzyme complexes for the substrates, factors X and prothrombin,  
respectively (Reference 42). The membranes also serve as allosteric  
10 activators of the enzyme-cofactor complex (Reference 29). PS-containing  
membranes also support anticoagulant activity that modulates the  
procoagulant activity. For example, protein C is activated by the thrombin-  
thrombomodulin complex on PS-containing membranes (Reference 43) and  
activated protein C inactivates factor Va and factor VIIIa on PS-containing  
15 membranes (Reference 44). The membranes of quiescent blood cells do not  
display the PS necessary to enable assembly and function of the  
procoagulant enzyme complexes (References 45-46). Rather, it is exposed  
only after cells are stimulated or undergo apoptosis (Reference 47). In the  
setting of a tissue injury, the procoagulant membranes are probably on the  
20 surface of platelets that have adhered to the damaged tissues (Reference 48).  
The absence of a PS-containing phospholipid membrane effectively prevents  
function of the complexes. Thus, blocking the PS-containing phospholipid  
binding sites on platelets appears to be a potential mechanism for preventing

blood coagulation or altering the procoagulant/anticoagulant balance. Additional investigation is recommended to determine whether lactadherin has this physiologic function.

5           **[0080]**       Several proteins have been identified that can influence blood coagulation via interaction with phospholipid membranes. The hypothesis that lactadherin might function in this manner was based on the homology between the discoidin-type domains of lactadherin and those of factors VIII and V, together with the previously defined membrane-binding  
10   properties of lactadherin (Reference 6). Annexin V, the most thoroughly studied of these proteins, binds to PS-containing membranes with high affinity (Reference 25). However, annexin V binds poorly to curved membranes (Reference 40) requires supraphysiologic  $\text{Ca}^{++}$  concentrations for optimal binding, and inhibits less than 80% of procoagulant function on endothelial  
15   cell membranes unless the concentration exceeds 200 nM (Reference 49). Likewise, annexin V is an incomplete inhibitor of the factor Xase complex on platelet membranes (Reference 50). Our results, indicating inhibition of the prothrombinase complex exceeds 80% at 60 nM annexin V only when the curvature of the membrane is minimal and the PS content is 15%, are in  
20   agreement with these prior studies.  $\beta_2$ -glycoprotein I binds to PS-containing membranes and other negatively charged lipid-containing particles such as lipoproteins. Purified  $\beta_2$ -glycoprotein I partially inhibits prothrombinase activity on purified platelets or phospholipid vesicles (Reference 51). However,  $\beta_2$ -

glycoprotein I may be a more efficient inhibitor of the anticoagulant reaction in which activated protein C cleaves factor V or factor Va on a phospholipid membrane (Reference 52).  $\beta_2$ -glycoprotein I bound to phospholipid is the primary antigen of lupus-type anticoagulants (Reference 53). When an  
5 antibody links two  $\beta_2$ -glycoprotein I molecules the membrane-binding affinity is increased and  $\beta_2$ -glycoprotein becomes a more potent *in vitro* anticoagulant (Reference 54). Whether  $\beta_2$ -glycoprotein I has a physiologic function influencing procoagulant or anticoagulant membrane interactions remains unknown (Reference 55). The physiologic relationship of these proteins and  
10 lactadherin to blood coagulation is a likely field for further investigation.

**[0081]** Factor VIII binds to sites on phospholipid membranes with remarkable specificity. The specificity is best illustrated by the failure of other lipid binding proteins to compete with factor VIII for these sites  
15 (Reference 16). Even factor V, with structural homology and equivalent affinity for phospholipid membranes competes for only a fraction of the sites recognized by factor VIII. The capacity of lactadherin to compete for membrane binding sites of factor VIII, also to inhibit both the factor Xase complex and the prothrombinase complex indicates that lactadherin is more  
20 promiscuous than factor VIII with regard to phospholipid binding sites (Reference 16). Inhibition of the factor VIIa-tissue factor complex indicates that lactadherin has the capacity to compete for the vitamin K dependent proteins, factor VIIa and/or factor X. The contrast between lactadherin and



annexin V with regard to competing for membrane sites of both high and low PS content and varying membrane curvature indicate that lactadherin is also more promiscuous in its membrane requirements than annexin V. To facilitate understanding of these properties we have initiated studies to  
5 characterize the membrane binding properties of lactadherin vs. those of factor VIII and factor V. The results indicate that lactadherin resembles factor VIII and factor V in specific binding to PS and curvature-dependent membrane binding. However, lactadherin differs in having a lower PS requirement and no apparent requirement for PE on membranes with low PS content.

10

[0082] Comparing the displacement of factor VIII and factor V by lactadherin with the competition predicted from the simplest mathematical model (Figures 1A-C and 3A-F) supports two conclusions. First, lactadherin is a better competitor for factor VIII binding sites than for factor V binding sites  
15 with a  $K_i$  that is two-fold lower. More potent competition for factor VIII binding sites correlated with a two-fold lower  $K_i$  for the factor Xase complex vs. the prothrombinase complex not only under conditions where phospholipid was limiting but also when phospholipid was not limiting (Figure 6). The lower  $K_i$  value suggests that lactadherin binds with higher affinity to phospholipid  
20 binding sites of factor VIII vs. those of factor V. The second conclusion is that the experimental data did not precisely conform to the curves predicted by the mathematical model. The assumption of the model was that lactadherin competed for a single class of phospholipid binding sites with factor VIII or

factor V. In other studies, we have further characterized the interaction of lactadherin with phospholipid binding sites. Our results indicate that lactadherin recognizes at least 2-classes of phospholipid binding sites so that both association and dissociation are kinetically complex events. The ability  
5 to interact with multiple classes of phospholipid binding sites explains part of the variation from the simple model as well as the mechanism underlying the capacity to compete with factor VIII and factor V with different apparent  $K_i$ 's.

[0083] The concentrations of lactadherin required to inhibit the  
10 whole blood prothrombin time were somewhat higher than the concentrations necessary to inhibit isolated enzyme complexes (compare Figures 2-4 vs. Figure 6). Our data do not indicate whether these apparent discrepancies in concentration reflect lactadherin binding to a plasma protein that partially competes with binding sites on the membranes of platelets or other cells, or  
15 whether lactadherin may have a lower affinity for cell membranes vs. phospholipid vesicles.

[0084] The results of our experiments outlined above indicate that lactadherin could serve as a physiologic or pharmacologic anticoagulant.  
20 In newborn calves, the plasma concentration rises from 0.07  $\mu\text{g/ml}$  before feeding to 1.2  $\mu\text{g/ml}$  after feeding, suggesting that intact lactadherin is absorbed across the GI tract and that sufficient lactadherin circulates in the

blood under these conditions to have a measurable *in vitro* anticoagulant effect (Reference 7). Lower concentrations of lactadherin have been measured in the serum of women with metastatic breast carcinoma but not in the serum of normal controls (Reference 56). The plasma levels in pregnant or lactating mammals have not been reported. However, it is plausible that sufficient lactadherin may be secreted into the blood or may "leak" from mammary glands to provide an anticoagulant effect during pregnancy or lactation. The relatively small size of lactadherin suggests that it freely traverses the placental barrier and could affect the procoagulant/anticoagulant balance of a developing fetus. The presence of lactadherin on the apical surfaces of secretory epithelia other than breast tissue suggests that lactadherin may circulate in blood even during the non-pregnant state. Thus, it is plausible that lactadherin could provide a physiologic anticoagulant function under a variety of circumstances. Our data support the conclusion that lactadherin is a candidate for development as a pharmacologic anticoagulant. Lactadherin functions at steps that are early in the coagulation pathway and is apparently more potent than annexin V, the only other tested agent that functions by a similar mechanism.

20       **[0085]**       In view of the above, we believe that:

- a.       Lactadherin may be used as an anticoagulant in human beings.  
Because of its unique mechanism of action it may prove useful

in specific clinical situations such as diffuse intravascular coagulation of obstetrics or septic shock. The natural method in which it is produced by mammary glands and secreted in milk suggests that it would prove much cheaper to produce than most protein drugs. Depending upon its efficacy, it might have a market as broad as all pregnant women with blood clots or patients with atherosclerosis who are experiencing unstable angina or a heart attack.

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- b. Lactadherin may be used as an anti-inflammatory agent in human beings, particularly in circumstances where blood coagulation is associated with inflammatory reactions including cell signaling mediated by coagulation enzymes and tissue factor. An example of a clinical situation where lactadherin may have anti-inflammatory properties is in sepsis.

15

- c. Lactadherin may be used to measure procoagulant phospholipid that circulates in the blood of patients. Because lactadherin completely blocks access to procoagulant phospholipid, it may be applied to laboratory assays as a method to block access of blood coagulation proteins from all circulating procoagulant phospholipids and to determine the residual coagulation activity.

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- d. Lactadherin may be used as a laboratory reagent to remove circulating phospholipids from the plasma of patients in order to measure the coagulation potential of plasma in the presence of

defined phospholipid. For example, lactadherin might be covalently linked to a matrix such as sepharose and procoagulant lipids removed by passage over the matrix. It might then be possible to provide a sensitive assay for lupus-type anticoagulants.

5

- e. Measurement of blood lactadherin concentrations may be useful to guide anti-coagulant or anti-inflammatory therapy. For example, lactadherin may be a natural anticoagulant during pregnancy, lactation, or neonatal life. Measurement may predict risk of thrombosis or guide therapy in case of thrombosis.

10

**[0086]** A pharmaceutical composition including lactadherin or a fragment of lactadherin, or a functionally equivalent agent thereof, may be prepared, in a conventional manner. In particular, a pharmaceutical composition made in accordance with the present invention would include lactadherin or a fragment of lactadherin, or a functionally equivalent agent thereof, in an amount sufficient to provide therapeutic and/or prophylactic benefit, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Compositions of the present invention may be formulated for any appropriate manner for administration, including, for example, oral, nasal, intravenous or intramuscular administration. Appropriate dosages, duration and frequency of administration would be determined by known factors, such as the condition

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of the patient, the type and severity of the disease and the method of administration.

**[0087]** While this invention has been described as having  
5 preferred ranges, steps, materials, or designs, it is understood that it is  
capable of further modifications, uses and/or adaptations of the invention  
following in general the principle of the invention, and including such  
departures from the present disclosure as those come within the known or  
customary practice in the art to which the invention pertains, and as may be  
10 applied to the central features hereinbefore set forth, and fall within the scope  
of the invention and of the limits of the appended claims.

**[0088]** The following references, to the extent that they provide  
exemplary procedural or other details supplementary to those set forth herein,  
15 are specifically incorporated herein by reference.

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## WHAT IS CLAIMED IS:

1. A method of blocking or reducing access to a procoagulant molecule by a coagulation molecule, comprising;

subjecting a procoagulant molecule to lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.

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2. The method of Claim 1, wherein:

a cell membrane or a fragment thereof comprises the procoagulant molecule.

3. The method of Claim 1, wherein:

the procoagulant molecule comprises a phospholipid or a lipoprotein.

4. The method of Claim 3, wherein:

the coagulation molecule comprises a blood coagulation protein.



5. The method of Claim 3, wherein:  
the coagulation molecule is selected from the group consisting of factor V, factor Va, factor VIIa, factor VIII, factor VIIIa, factor IXa, and factor X.
6. The method of Claim 4, wherein:  
the procoagulant molecule comprises phosphatidylserine (PS).
7. A method of blocking or reducing binding of a ligand to a cell membrane binding site, comprising:  
subjecting a cell membrane binding site to lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.
8. The method of Claim 7, wherein:  
the ligand comprises a blood coagulation molecule selected from the group consisting of factor V, factor Va, factor VIIa, factor VIII, factor VIIIa, factor IXa, and factor X.
9. The method of Claim 8, wherein:  
the cell membrane site comprises a phospholipid.

10. A method of inhibiting or slowing blood coagulation, comprising:  
subjecting a predetermined amount of blood to an  
effective amount of lactadherin, a fragment of lactadherin, a  
functional equivalent of lactadherin, or a functional equivalent of  
a fragment of lactadherin.

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11. A method of inhibiting or slowing blood clotting, comprising:  
administering to a subject in need thereof an effective  
amount of lactadherin, a fragment of lactadherin, a functional  
equivalent of lactadherin, or a functional equivalent of a  
fragment of lactadherin.

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12. A method of preventing or reducing inflammation, comprising:  
administering to a subject in need thereof an effective  
amount of lactadherin, a fragment of lactadherin, a functional  
equivalent of lactadherin, or a functional equivalent of a  
fragment of lactadherin.

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13. A method of inhibiting or slowing blood coagulation, comprising:  
administering to a subject in need thereof an effective  
amount of lactadherin, a fragment of lactadherin, a functional  
equivalent of lactadherin, or a functional equivalent of a  
fragment of lactadherin.

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14. An anticoagulant reagent, comprising:
- lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, or a functional equivalent of a fragment of lactadherin.
15. A pharmaceutical composition, comprising the reagent of Claim 14 and a pharmaceutically acceptable carrier or diluent.
16. A method of removing a phospholipid from a biological fluid, comprising:
- a) providing a biological fluid including, or suspect of including, a phospholipid;
- b) 5     subjecting the biological fluid to a suitable amount of a binding agent selected from the group consisting of lactadherin, a fragment of lactadherin, a functional equivalent of lactadherin, and a functional equivalent of a fragment of lactadherin;
- c) 10     allowing binding between the phospholipid and the binding agent; and
- d)     removing the binding agent with any phospholipid bound thereto.

17. The method of Claim 16, wherein:  
the binding agent is provided in a sepharose matrix.
18. A kit for removing a phospholipid from a biological fluid,  
comprising:
- a) a binding agent selected from the group consisting of  
lactadherin, a fragment of lactadherin, a functional  
equivalent of lactadherin, and a functional equivalent of a  
fragment of lactadherin; and
- b) instructions for use of the binding agent.
19. The kit of Claim 18, further comprising:  
a sepharose matrix comprising the binding agent.

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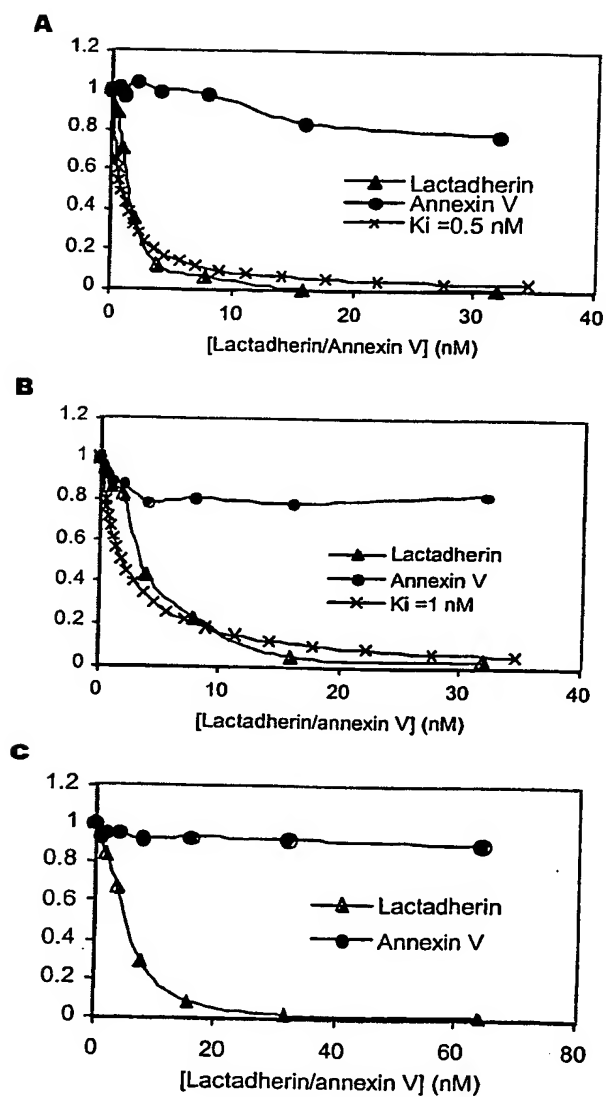


Figure 1

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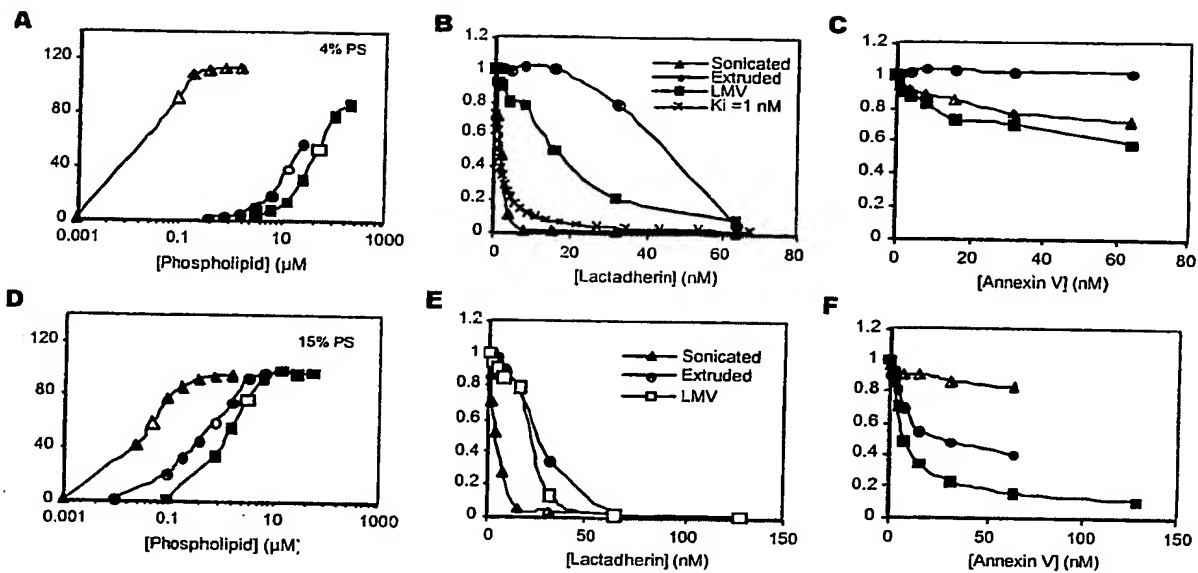


Figure 2

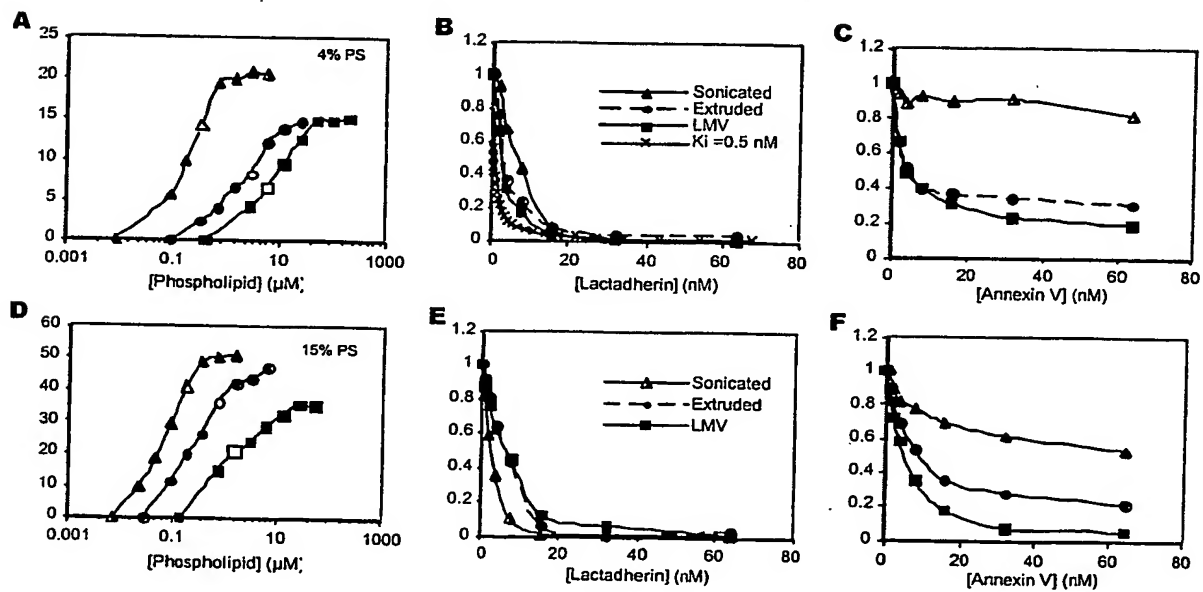


Figure 3

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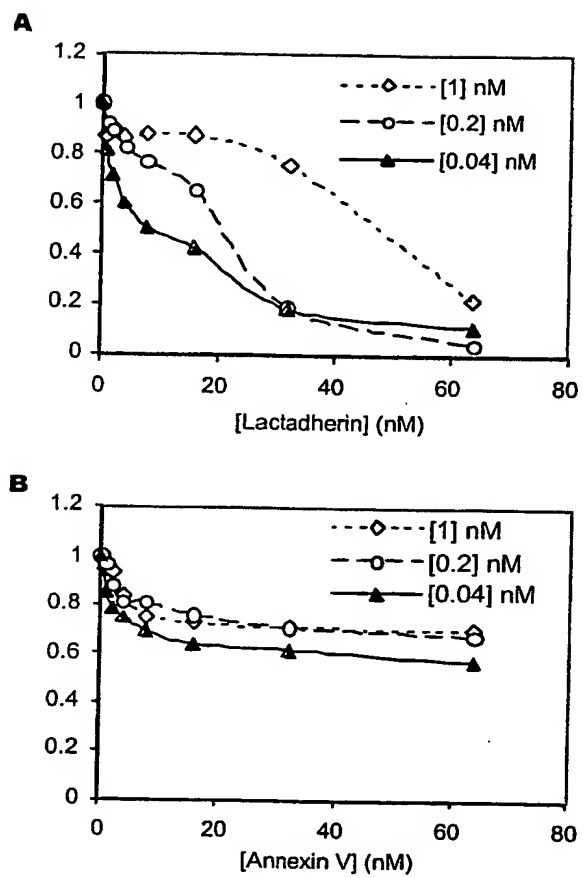


Figure 4

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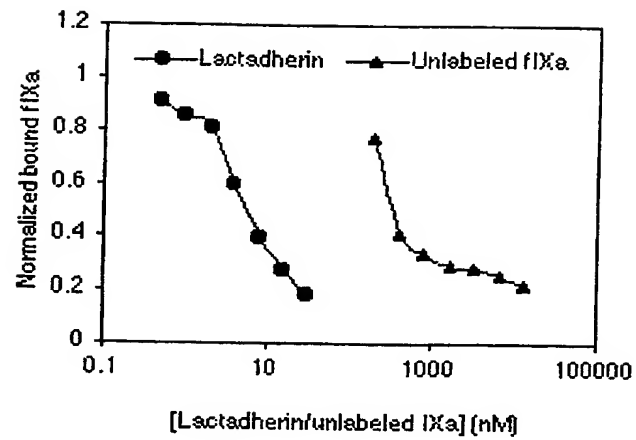


Figure 5



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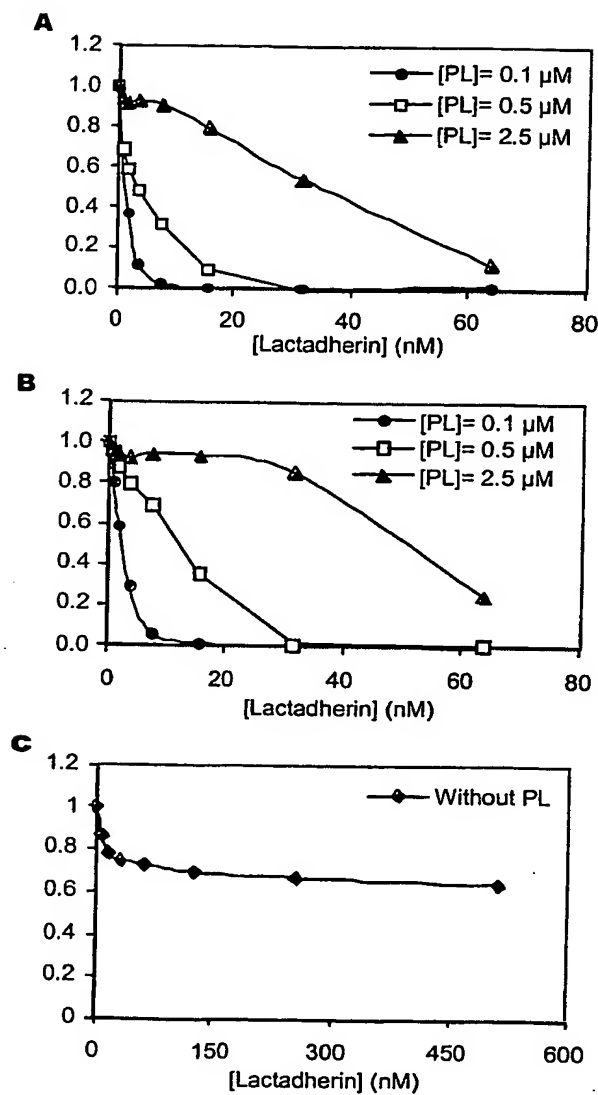


Figure 6

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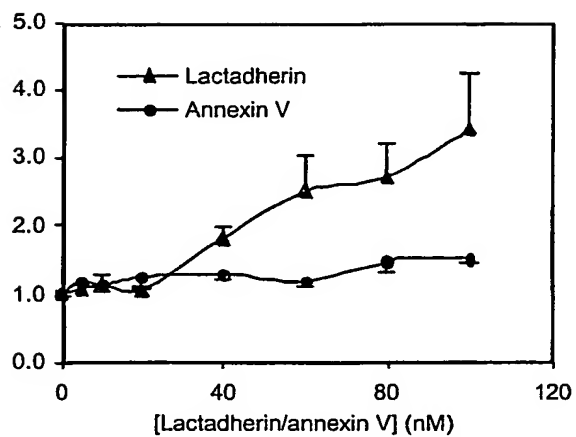


Figure 7

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/15404

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : A61K 38/16, 38/36

US CL : 514/8

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/8;435/6, 69.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ORTEL et al. Deletion analysis of recombinant human factor V. Evidence for a phosphatidylserine binding site in the second C-type domain. J. Biol. Chem. 25 February 1992, Vol. 267, No. 6, pages 4189-4198. See page 4189 and discussion, "...human factor V, similar to human factor VIII, possesses a phosphatidylserine-specific binding site within the C2 domain of the light chain."	1-19
Y	LARocca et al. A Mr 46,000 human milk fat globule protein that is highly expressed in human breast tumors contains factor VIII-like domains. Cancer Res. 15 September 1991, Vol. 51, No. 18, pages 4994-4998. See page 4997, "The nucleotide and deduced amino acid sequences of BA46-1 cDNA are shown...the deduced protein sequence, however, shares identity with factors V and VIII." "The homology with clotting factors is in the C1,C2 region of the light chain of factor VIII."	1-19
Y	ORTEL et al. Characterization of an acquired inhibitor to coagulation factor V. Antibody binding to the second C-type domain of factor V inhibits the binding of factor V to phosphatidylserine and neutralizes procoagulant activity. J. Clin. Invest. 1992, Vol. 90, pages 2340-2347. See page 2341, "...this inhibitor neutralizes the procoagulant activity of Factor Va by interfering with the C2-domain mediated binding to anionic phospholipids..."	1-19



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

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later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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document member of the same patent family

Date of the actual completion of the international search

12 August 2003 (12.08.2003)

Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

PCT/US03/15404

## C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P --- X,P	US 2003/0022221 A1 (LANGIT et al.) 30 January 2003 (30.01.2003), page 3, column 1, paragraphs 29, and 30. "The present invention provides nucleic acid sequences that encode protein molecules...related to the lactadherin protein subfamily..The peptide sequences provided..as well as the obvious variants described herein.."	1-13, 16-19 ----- 14,15